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# NITRIC OXIDE SYNTHASES: WHY SO COMPLEX?

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## CONTENTS



## INTRODUCTION

This review does not attempt to present a balanced or comprehensive view of the abundant literature on the biology and chemistry of nitric oxide. Instead, it is based on 30 years of dabbling in the biochemistry of those enzymes that bear derivatives of riboflavin or protoporphyrin IX as prosthetic groups, i.e. flavoproteins and heme proteins. Since the late 1960s, we have known of enzymes from bacterial sources (*Escherichia coli* and *Salmonella typhimurium*) that contain both flavin and heme as prosthetic groups as well as other redox cofactors, namely the NADPH-mediated sulfite reductases studied by Siegel & Kamin and their colleagues (44, 45, 48, 64). These enzymes contain an unusual heme known as siroheme (44, 45), and both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) were present in stoichiometric amounts (64, 48). Fulco and his coworkers (46, 47) and Peterson et al (3, 60) later isolated and studied a soluble, catalytically self-sufficient protein from *Bacillus megaterium* that contained heme, FAD, and FMN in equimolar ratios and that required NADPH as a source of electrons. Until the discovery



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of nitric oxide synthase (NOS) isoforms, enzymes containing both flavin and heme prosthetic groups in the same polypeptide had not been identified in mammalian systems. In addition, NOSs from various organs and cell types contain tetrahydrobiopterin and exhibit consensus sequences for calmodulin binding, a unique property for a redox enzyme. This review examines the enzymology and structural attributes of the various NOSs that make them uniquely suitable for their systemic functions as producers of neurotransmitters, endothelium relaxing factor(s), or cytotoxic agents, all under the guise of the diatomic molecule, nitric oxide (NO•).



## THE BIOLOGY OF NITRIC OXIDE AND NITRIC OXIDE SYNTHASES

In 1916, Mitchell et al found that the amount of urinary nitrogen oxides ($NO_3^-$) exceeds that derived from the diets of rats, pigs, and humans (43), but proof for such metabolism was not forthcoming until 1981, when Tannenbaum and colleagues used stable $^{15}N$-labeled precursors and found labeled nitrate metabolites (19, 20). In further studies, Stuehr & Marletta (69) concluded that *E. coli*–derived lipopolysaccharide stimulated in vitro production of nitrite ($NO_2^-$) and nitrate ($NO_3^-$) by macrophages. At the same time, the respective laboratories of Marletta (25) and Hibbs (23) found that the ultimate source of these nitrogen oxides (subsequently called $NO_x$) was the amino acid L-arginine. The nitrogen of NO• is derived from the enzymatic oxygenation of one of the two guanidino nitrogens of L-arginine, and L-citrulline is formed from the remaining carbon backbone of L-arginine. This review discusses the biochemical mechanism of this process (vide infra).

Between 1977 and 1979, four groups (1, 12, 21, 40) proposed that NO• stimulates guanylate cyclase activity, thus accounting for its vasodilatory properties. Following the observation of Furchgott & Zawadski (15), three groups led by Moncada, Ignarro, and Furchgott (14, 24, 51), respectively, reported that NO• production could account for endothelium-derived relaxation. A recent review by Stuehr & Griffith outlines these developments in tabular form (66).

Garthwaite et al (16) subsequently implicated this seemingly insignificant diatomic molecule in the function of the central nervous system (CNS). Shortly thereafter, Moncada's laboratory (17) found NO•-producing enzymatic activity in rat brain slices. In 1989, Bredt & Snyder (7) examined the role of NO• production in the brain by demonstrating that glutamate and structurally related amino acids, such as N-methyl-D-aspartate (NMDA), markedly stimulate the conversion of arginine to citrulline in cerebellar slices concomitant with the increase in cGMP levels. At the same time, Moncada's group (29) reported the concomitant $Ca^{2+}$ dependence of guanylate cyclase activation and L-citrull-

ine synthesis in synaptosomal cytosols. Knowles et al (29) found that the synthesis of both NO• and L-citrulline by the synaptosomal supernatants was fully dependent on $Ca^{2+}$, with half-maximal rates obtained at ~ 160–170 nM for both products. In fact, the formation of NO• (measured as guanylate cyclase stimulation) was absent at resting physiological concentrations of free $Ca^{2+}$ (~ 80 nM) in synaptosomes but fully active at > 400 nM $Ca^{2+}$. Furthermore, Bredt & Snyder (7) showed that $N^G$-monomethyl-L-arginine (L-NMA) blocked the formation of L-citrulline and cGMP with equal potency and that L-arginine reversed this inhibition. Although data were not shown, the findings of these authors (7) corroborated the data of Knowles et al (29), which stated that the conversion of [$^3$H]arginine to [$^3$H]citrulline in cerebellar homogenates was fully dependent on NADPH and added $Ca^{2+}$. Bredt & Snyder (7) also demonstrated that hemoglobin, which complexes NO•, blocks stimulation of cGMP levels by N-methyl-D-aspartate and that superoxide dismutase, which elevates NO• levels, increases the levels of cGMP. From this study they concluded that NO• mediates the stimulation of cGMP formation at the level of glutamate receptors (7).

Meanwhile, Palmer & Moncada (52), who had previously demonstrated (50) that L-arginine was the source of endothelium-derived NO• and L-citrulline, purified a novel citrulline-forming enzyme from homogenates of porcine aortic endothelial cells. In a subsequent publication, Bredt & Snyder (8) reported the isolation of NOS as a calmodulin-requiring enzyme that could form stoichiometric amounts of L-citrulline from L-arginine in the presence of NADPH and $Ca^{+2}$. The purification process used the 2′,5′-ADP-Sepharose 4B biospecific affinity chromatography procedure employed by Yasukochi & Masters (74) in 1976 to purify liver microsomal NADPH-cytochrome P450 reductase (see below). Although the preparation was unstable at 0° C (50% degradation in 2 h), Bredt & Snyder (8) could determine the $K_m$ for L-arginine as substrate (~ 2 μM) and the $EC_{50}$ for calmodulin enhancement of L-citrulline production (10 nM). Analysis of the final preparation on 7.5% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) showed a single band, but the protein yield from 18 rat cerebella was a scant 9 μg. However, as Bredt & Snyder correctly pointed out (8), sufficient enzyme could now be purified to obtain antisera. They proposed molecular cloning as a means to ascertain the amino acid sequence for detailed comparisons with similar enzymes.

In subsequent immunohistochemical localization studies, Bredt et al (6) found NOS associated with discrete brain regions, cell types, and cellular processes. They observed variations in immunoreactivity in rat brain by using affinity-purified antiserum to purified rat brain NOS as the primary antibody and an avidin-biotin-peroxidase system as the secondary antibody, with diaminobenzidine as a chromogen. High immunoreactivity occurred in the cerebellar molecular and granule cell layers, olfactory bulb granule cell layer,

superior and inferior colliculi, dentate gyrus of the hippocampus, diagonal band of Broca, and posterior lobe of the pituitary gland. Intense staining was also seen in peripheral neural systems, particularly in the myenteric plexus of the intestinal tract, retina, and adrenal medulla and in the endothelial layers of large blood vessels. These findings were consistent with reports of NOS activity in endothelial cells (14, 24, 51). Interestingly, the antibodies to the rat cerebellar NOS did not cross-react with the inducible macrophage NOS, which further substantiates the distinct natures of these isoforms (6).



## CHARACTERIZATION OF NITRIC OXIDE SYNTHASES

### *Biochemistry*

Nomenclature systems for NOSs from various organs are related to localization and/or response to stimuli. A basal level of endogenous nitrate biosynthesis from L-arginine occurs in rodents and humans (32). Immunostimulation of nitrate biosynthesis was also observed in rodents and humans (71). Stuehr & Marletta (70) later demonstrated that this immunostimulation was mimicked by bacille Calmette-Guérin (BCG) infection, lymphokines, and interferon-γ in mouse macrophages. While this has been a convenient operational categorization, it is not useful or meaningful to assume that only one type of nitric oxide synthase occurs within a single cell type (59). The experimental conditions under which such immunostimulation is performed are important in defining isoforms involved in any given tissue or cell type, e.g. in cell culture in which the medium may be so restricted as to exclude essential nutrients required to maintain the complement of cofactors.

Ultimately to understand the complex mechanism(s) that control(s) NO• formation, investigators attempted to purify the various NOSs from cerebellar, macrophage, and endothelial sources. Several laboratories published the first successful procedures in 1990 and 1991 following the initial purification and partial characterization of rat cerebellar NOS by Bredt & Snyder (8). Although these authors were the first to determine the calmodulin requirement and define kinetic parameters such as the $K_m$ for L-arginine (1.5 μM), the $K_i$ for L-NMA (1.4 μM), and an $EC_{50}$ of 10 nM for calmodulin, this study did not establish any other cofactor requirements. Meanwhile, Mayer et al (38) purified a $Ca^{2+}$- and calmodulin-dependent NOS from porcine cerebellum and described a cofactor role for tetrahydrobiopterin. They also reported (38) a native molecular mass of 200 kDa using gel permeation chromatography. In the following year, Schmidt et al (62) purified rat cerebellar guanylyl cyclase–activating-factor synthase to electrophoretic homogeneity and determined its subunit molecular weight to be 155 kDa on 7.5% SDS/PAGE. Their purification utilized the 2′,5′-ADP-Sepharose 4B affinity chromatography procedure em-

ployed by Bredt & Snyder (8) and a calmodulin-agarose affinity column. This enzyme preparation exhibited activity with L-arginine as substrate ($K_m \sim 2.2$ μM), was inhibited by $N^G$-nitro-L-arginine ($IC_{50} \sim 0.9$ μM) and $N^G$-methyl-L-arginine ($IC_{50} \sim 1.6$ μM), and exhibited a $K_{0.5}$ for calmodulin of ~ 3.5 nM. Its guanylyl cyclase activation (cGMP content per $10^6$ cultured rat lung fibroblasts; RFL-6 cells) and citrulline-forming activities copurified.

Although Schmidt et al (63) reported a native molecular mass of 279 kDa by using a combination of sedimentation velocity and gel permeation techniques, Bredt & Snyder (8), using gel filtration chromatography on Superose-6, reported an apparent molecular mass of ~ 200 kDa for the rat cerebellar NOS that corroborated the results of Mayer et al (38) on the porcine cerebellar enzyme. Our recent experiments on cloned, expressed rat cerebellar NOS utilizing analytical ultracentrifugation techniques (E Sheta, P Schwartz, K McMillan, JC Hansen, BSS Masters, unpublished observations) indicate that the enzyme exists in a monomer-dimer equilibrium that tends toward increasing monomer concentration at decreasing enzyme concentration (lower limit $S_{20,w}$ of 4.7), determined in sedimentation equilibrium studies. (The presence of $Ca^{2+}$-calmodulin shifts this equilibrium toward the dimer.)

Efforts to purify and characterize the various isoforms of NOS continued. Using a cytomegalovirus promoter, Bredt et al (5) reported the cloning and expression of a full-length rat cerebellar NOS in human kidney 293 cells. They assayed NOS activity in transfected cells by three methods: (*a*) the enzymatic conversion of [$^3$H]arginine to [$^3$H]citrulline, (*b*) the generation of nitrite from cold L-arginine, and (*c*) the enhancement of endogenous guanylyl cyclase activity in response to NO•. The authors reported 10-fold greater activity in NOS cDNA-transfected kidney 293 cells than in cerebellar extracts, the most abundant source of constitutive brain-type NOS.

Bredt et al (5) reported a number of recognition sites relevant to the function of the enzyme. First, there is a basic amphipathic α helix calmodulin-binding consensus sequence between positions 725 and 745, consistent with its calmodulin requirement. A consensus sequence for cAMP-dependent protein kinase–mediated phosphorylation begins at position 473, but no consensus sequences for phosphorylation by protein kinase C or $Ca^{2+}$/calmodulin protein were identified. A NADPH-binding domain is discernible between residues 1204 and 1429, with sites for contact of the adenine and ribose rings at positions 1245–1263 and 1343–1358, respectively, in close homology with NADPH-cytochrome P450 reductase, sulfite reductase, and ferredoxin-$NADP^+$ reductase as described by Porter & Kasper (56). In addition, Bredt et al suggest (5) that because (*a*) tetrahydrobiopterin stimulates the macrophage and porcine brain NOSs and (*b*) consensus-binding sequences are present for FAD and FMN, this (these) enzyme(s) could have other cofactor requirements. They reported the stoichiometric content of FAD and FMN in their purified rat brain

preparation but did not present supporting data. Nevertheless, this publication laid the groundwork for the production of amounts of cerebellar NOS sufficient to permit definitive characterization using physicochemical approaches.

Experiments by Mayer et al (39) indicated that one mole each of FAD and FMN were bound per mole of porcine cerebellar NOS and that their preparations contained 0.7–1.11 atoms of iron per mole of enzyme. They therefore concluded that brain NOS is a nonheme iron protein. Unfortunately, no mention was made of attempts to determine whether the preparations contained heme.



In contrast, when Schmidt et al (62) purified and analyzed the rat cerebellar NOS for binding of NADPH, FAD, and FMN, they found only one mole each of these cofactors per dimer, which indicated to them that cofactors are shared between identical subunits. These authors also suggested that the absence of biopterins other than the fully reduced form, tetrahydrobiopterin ($BH_4$), could explain the lack of inhibitory effect of methotrexate on enzyme activity. However, studies in other laboratories (4, 5, 39) do not support this stoichiometry for flavin binding to the cerebellar NOS. Moreover, Marletta's laboratory (22) and Stuehr et al (65) reported that macrophage NOS contains one mole each of FAD and FMN per 130 kDa subunit.

In the meantime, the Murad laboratory continued their studies on the purification and characterization of the particulate endothelium-derived relaxing factor (EDRF) synthase, now universally called NOS, from cultured and freshly isolated bovine aortic endothelial cells (54, 55). In these studies, Pollock et al (54, 55) reported the properties of particulate and soluble forms of endothelial NOS. The purified particulate endothelial NOS had a denatured molecular mass of 135 kDa on 7.5% SDS/PAGE. Stimulation of cGMP production in RFL-6 cells by purified endothelial enzyme was $Ca^{2+}$ and calmodulin-dependent (55). In addition, the following kinetic parameters were determined for the purified particulate form: $K_m$ for L-arginine = 2.9 μM, $EC_{50}$s for $BH_4$ = 0.1 μM, $Ca^{2+}$ = 0.3 μM, and calmodulin = 3.5 nM. The $K_i$ values for $N^G$-methyl-L-arginine and $N^G$-nitro-L-arginine are 0.94 and 0.16 μM, respectively. Therefore, both endothelial forms (54), as well as the rat brain form (8), are dependent on $Ca^{2+}$/calmodulin for activity. Having demonstrated that both particulate and cytosolic forms of calmodulin-dependent EDRF/NOS activity occur in bovine aortic endothelial cells (13), Murad's group went on to elucidate the differences in chromatographic and immunochemical behavior between these two bovine aortic endothelial forms (Type III NOSs) and the soluble rat brain form (55; Type Ia).

Of interest in this regard is the report by Michel's laboratory of a consensus motif for N-terminal myristoylation, which is absent in the primary sequences of the soluble macrophage and neuronal NOSs (31). These investigators performed oligonucleotide-directed mutagenesis of the myristoylation consensus

sequence in the endothelial NOS and examined the effect of these mutations on the localization of expressed enzyme in COS-7 (11). Expression of wild-type endothelial NOS cDNA in COS-7 cells localized both enzymatic activity and NOS immunoreactivity to the particulate fraction, whereas transient expression of myristoylation-minus mutant cDNA localized both enzymatic activity and immunoreactive protein to the cytosol. Michel et al suggested that this N-terminal myristoylation, which is unique to the endothelial NOS, may play a regulatory role. They also examined the phosphorylation of intact, cultured bovine endothelial cell NOS (42) in light of the earlier observations of Bredt et al (5) on the recognition sequences for phosphorylation on rat brain NOS. The data obtained by Michel et al (42) indicate that endothelial NOS undergoes phosphorylation in response to bradykinin, resulting in the translocation of the enzyme from the particulate to the cytosolic compartments. The authors propose that this translocation leads to deactivation of the protein. In fact, the induction of phosphorylation by sodium nitroprusside may relate to the observations by Ignarro's group (61, 10) that NO• inhibits neuronal and endothelial NOS isoforms.



## *Mechanism*

The reaction sequence for the formation of citrulline and NO• from L-arginine requires the incorporation of one atom of oxygen into each of the products formed. $^{18}O_2$ experiments in a variety of systems (30, 33) determined that the source of these oxygen atoms is molecular $O_2$ for the formation of both NO• and L-citrulline. Stuehr et al (68) and, later, Pufahl et al (58) reported that $N^G$-hydroxy-L-arginine (L-NHA) is an intermediate in the biosynthesis of NO• from L-arginine. Stuehr et al (68) also observed that 1.5 and 0.5 mole of NADPH were consumed in the formation of $NO_2^-$ and $NO_3^-$ from L-arginine and (L-NHA), respectively. These experiments were performed in both the absence and the presence of substrate, and the substrate-specific fraction of NADPH oxidation was calculated under the assumption that such a fraction represented the proportion of added NADPH used for NO• synthesis. This assumption has been accepted in proposed mechanisms (66, 58) and forms the basis for the mechanism presented in this review.

Despite reports of nonheme iron content in NOSs, reports of sequence homology of the C-terminal 641 amino acids [36% sequence identity; 58% homology (5)] of the cloned rat cerebellar NOS to microsomal NADPH-cytochrome P450 reductase prompted investigators to examine the structure of these proteins for bound heme. In 1992, a number of research groups reported stoichiometric amounts of CO-binding heme in NOSs from mouse macrophages (72), from rat cerebellar NOS expressed in human kidney 293 cells (41a, 67), from mouse macrophage cell line RAW 264.7 (67), and from porcine cerebellum (28). These reports also noted that the heme of NOSs from brain

and macrophage sources bound CO when the enzyme was reduced. Interestingly, the absorption maximum for the reduced, CO difference spectrum of NOSs occurs in the 443–448 nm wavelength region, reminiscent of the cytochromes P450. Such a resemblance was unexpected, since data bank searches had failed to reveal sequence homologies between the several hundred cloned cytochromes P450 and the NOSs. Nevertheless, McMillan et al (41a) suggested a consensus sequence surrounding a cysteinyl peptide region in the N-termini as the heme-binding peptide of the NOSs from three tissue sources: rat brain NOS (5), RNASRC$^{415}$VGRIQW; murine macrophage NOS (73), RNAPRC$^{194}$IGRIQW; bovine endothelial NOS (31), RNAPRC$^{186}$VGRIQW. These sequences bear little resemblance to those found in members of the cytochrome P450 gene family.

All of the previously mentioned reports of heme content (28, 41a, 67, 72) either presented data or stated that CO inhibited by 70–80% the catalysis of L-citrulline formation from L-arginine by purified or partially purified preparations. These reports, together with additional data that showed that miconazole—a known inhibitor of cytochromes P450—also inhibited L-citrulline formation catalyzed by NOS (28), led to the implication of bound heme in the oxygenation of L-arginine in the reaction mechanism. Recently, Pufahl & Marletta (57) examined the involvement of heme in catalysis of $NO_2^-$ and $NO_3^-$ formation by macrophage NOS, using L-arginine and $N^G$-hydroxy-L-arginine (L- NHA, the first hydroxylated product and putative reaction intermediate) as substrates. These authors examined the relative inhibition by CO of macrophage NOS–mediated $NO_x$ formation and found that L-arginine metabolism was inhibited an average of 57%, whereas L-NHA conversion was inhibited only ~ 32%. An optical difference spectrum, obtained by adding L-NHA to oxidized macrophage NOS, produced a peak at 384 nm and a trough at 420 nm, results typical of Type I difference spectra obtained with certain substrates of cytochromes P450. Although no spectral binding constants could be determined from their data because only one concentration of L-NHA was added, these data nevertheless indicate that L-NHA interacts with the oxidized ($Fe^{+3}$) heme of NOS to produce a high-spin form.

Pufahl & Marletta (57) tested the capacity of L-NHA to reduce the heme in the absence of added reducing equivalents. The oxidized macrophage NOS was rendered anaerobic and placed under a CO atmosphere, at which time the intermediate was added. Because no reduced CO difference spectrum resulted, the authors concluded that $N^G$-hydroxy-L-arginine was not involved in the direct reduction of ferric heme iron. However, they did not preclude the involvement of the reaction intermediate in further reducing the ferrous-dioxygen complex (see Figure 1, 57). My laboratory (41b) has performed extensive experiments in which optical difference spectra were obtained with the cloned, expressed rat cerebellar NOS and the substrate (L-arginine), the intermediate

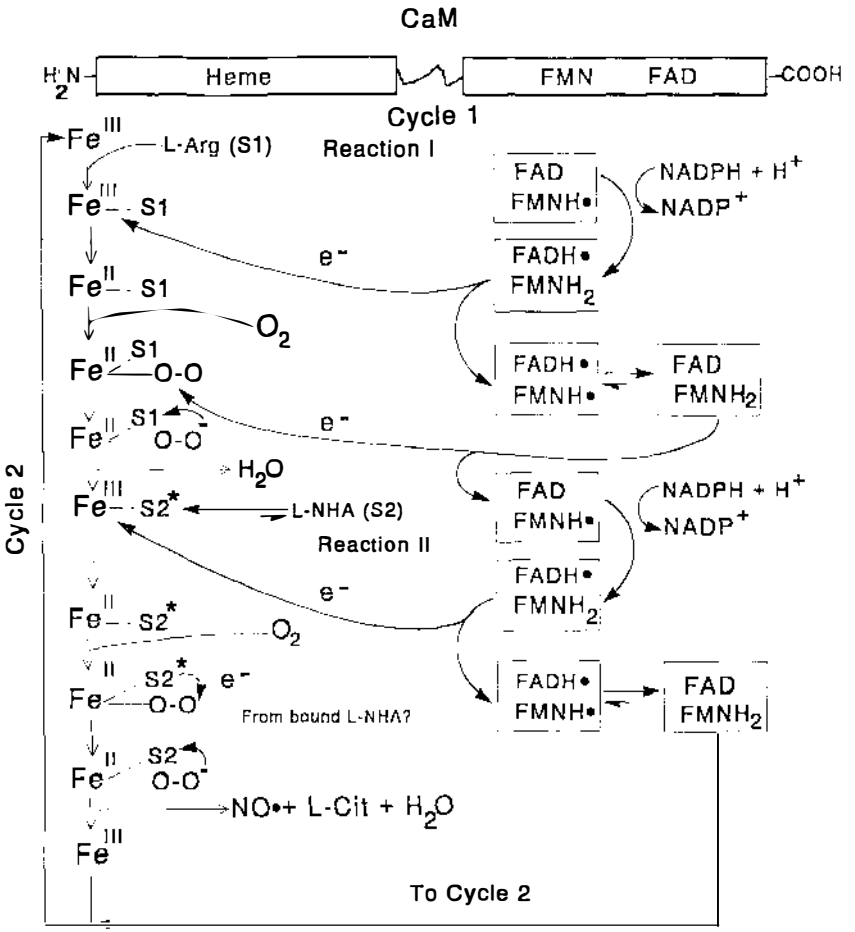


*Figure 1* The following assumptions are made for this scheme: (*a*) Based on the observations of Stuehr & Ikeda-Saito (67) that a flavin semiquinone free radical could be involved in brain NOS-catalyzed reactions, as we have demonstrated for NADPH-cytochrome P450 reductase (36, 37), the flavin intermediate FAD-FMNH• is shown (49). (*b*) The FAD-FMNH• form is considered the oxidized redox partner and is regenerated during catalytic turnover. The enzyme could require the $FMNH_2$ form to reduce various electron acceptors, as is the case for NADPH-cytochrome P450 reductase (36). (*c*) The stoichiometry of 1.5 moles of NADPH consumed per mole of L-citrulline formed (68) is accounted for by two cycles through this monooxygenation scheme. Integral stoichiometry is thereby preserved. (*d*) The overall reaction represents a 5-electron oxidation process in the formation of NO•.

L-NHA, the inhibitor ($N^G$-methyl-L-arginine), and the nitrogenous ligand imidazole. We (41b) concluded that the spectral binding constants were in excellent agreement with kinetically determined constants for L-arginine, L-NHA, and $N^G$-methyl-L-arginine. By introducing imidazole to the enzyme, we con-

verted the predominantly high-spin spectrum obtained with the freshly isolated NOS from kidney 293 cells to a typical low-spin spectrum; subsequent addition of substrate resulted in > 70% conversion to the high-spin state. These data (57, 41b) strongly indicate that the substrate, intermediate, and inhibitor can interact with the heme in its oxidized form. This interaction constitutes the first step in the reaction sequence.

The variable occurrence of tetrahydrobiopterin ($BH_4$) in numerous preparations of both brain and macrophage NOSs also suggested a role for this prosthetic group in a reaction sequence that involves the incorporation of one atom of oxygen into each of the products (L-citrulline and NO•). Recent experiments from Kaufman's laboratory (18) present convincing evidence that this cofactor does not function as a reactant in the oxygenation of L-arginine (reviewed in 9, 27, 35). Therefore, $BH_4$ likely plays an allosteric or stabilizing role in the reaction. This conclusion is based on the observations that $BH_4$ is effective at less than stoichiometric concentrations, is not recycled during the reaction, does not affect the initial reaction rate, and that enzyme activity is not inhibited by the addition of methotrexate.

Stuehr's laboratory (2) recently presented further evidence for this model of a stabilizing role for $BH_4$ in the association of the inactive monomeric subunits of macrophage NOS into functional homodimers. In these studies, Baek et al (2) demonstrated that monomeric and dimeric forms of macrophage NOS from interferon-γ- and lipopolysaccharide-stimulated RAW 264.7 cells could be separated by fast-performance liquid chromatography using anion exchange and gel filtration columns. Whereas the monomeric and dimeric forms contained the same amounts of FAD and FMN per mole of subunit as well as bound calmodulin, only the dimer contained detectable amounts of either heme or $BH_4$. Because the monomers were inactive with respect to $NO_2^-$ and $NO_3^-$ production (representing NO• formation) but were reconstitutable upon the addition of both heme and $BH_4$ and, optimally, in the presence of L-arginine, the authors proposed that these cofactors play a role in maintaining the active homodimer.

This evidence compels us to examine more closely the role(s) that bound heme may play in both monooxygenation reactions occurring within the complex polypeptide chain of NOS with all of its participating cofactors and prosthetic groups. Recently, mechanisms were envisioned that exclusively involve the heme of NOS in the substrate-binding site. These set the stage for subsequent redox chemistry (41b, 57, 35). Figure 1 presents a reaction scheme based on structural considerations of NOS, assuming that each of them will be demonstrated to be heme- and flavin-containing proteins with reaction mechanisms consistent with those demonstrated for microsomal electron transport systems (53).

Recent evidence supports a bidomain structure for the subunit of rat brain

NOS (E Sheta, K McMillan, BSS Masters, manuscript submitted; K McMillan, BSS Masters, unpublished observations). These experiments involve the use of limited proteolysis by immobilized trypsin, which results in carefully controlled fragmentation of the enzyme. Initial experiments showed a concomitant decrease in NOS activity and an increase in superoxide dismutase- insensitive NADPH-cytochrome *c* reductase activity. In the absence of calmod-ulin, ~ 89 kDa and ~ 79 kDa fragments (estimated by SDS/PAGE) can be obtained that, when separated under nondenaturing conditions, retain native structure to the extent that each fragment binds heme or FAD and FMN. The reductase fragment (C-terminus, ~ 79 kDa) catalyzes the reduction of cytochrome *c*, which is not inhibitable by superoxide dismutase, a property analogous to that of microsomal NADPH-cytochrome P450 reductase. In addition to retaining its heme-binding properties, as indicated by a reduced, CO difference spectrum with an absorbance maximum at ~ 445 nm, the heme fragment (N-terminus; ~ 89 kDa) exhibits a shift in wavelength to that typical of low-spin heme upon addition of imidazole and a subsequent shift indicative of formation of a high-spin form when L-arginine is added.

Limited proteolysis, performed in the presence of calmodulin, produced different fragmentation patterns, a result predicted by the location of the putative calmodulin-binding site between residues 725 and 745 (5). The resulting 89 kDa fragment binds heme, CO, and L-arginine, and the 79 kDa fragment catalyzes NADPH-cytochrome *c* reductase activity. In addition, large quantities of the heme domain (not including the calmodulin-binding sequences) that retains heme and binds L-arginine and the flavin-binding domain that catalyzes NADPH-cytochrome *c* reductase activity (K McMillan, BSS Masters, unpublished observations) have been expressed in *E. coli*. These results corroborate the proteolysis experiments and demonstrate that rat cerebellar NOS fragments of large molecular weight constitute domains that maintain structural integrity. The availability of large amounts of these soluble protein fragments will permit further structural analyses.

## CONCLUDING REMARKS

The efforts of investigators worldwide are only now beginning to elucidate the structure-function relationships of the various isoforms of NOS. Although nutritional deprivation or manipulation may well have a considerable impact on this enzyme, it is difficult to envision a state in which L-arginine levels, at least in the brain, could become limiting. An interesting line of study would be the effects of folic acid deprivation on neurotransmission and neural development. This is especially pertinent in light of recent clinical reports of infants with anencephaly resulting from folic acid deficiencies. The reader is referred

to Kaufman's recent review (27) for a discussion of possible roles for tetrahydrobiopterin in the catalysis of NO• formation by the various isoforms of NOS. Because the prosthetic groups (FAD and FMN) and the cofactor (NADPH) derive from riboflavin and niacin, respectively, other nutritional situations may impact the function of these important and highly complex enzymes in the various tissues in which they are found. Further investigations will certainly provide exciting and important insights into this field.





## *Literature Cited*

1. Arnold WP, Mittal CK, Katsuki S, Murad F. 1977. Nitric oxide activates guanylate cyclase and increases guanosine 3',5'-cyclic monoophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. USA* 74:3203–7
2. Baek KJ, Thiel BA, Lucas S, Stuehr DJ. 1993. Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J. Biol. Chem.* 268: 21120–29
3. Boddupalli SS, Oster T, Estabrook RW, Peterson JA. 1992. Reconstitution of the fatty acid hydroxylation function of cytochrome P-450$_{BM-3}$ utilizing its individual recombinant hemo- and flavoprotein domains. *J. Biol. Chem.* 267: 10375–80
4. Bredt DS, Ferris CD, Snyder SH. 1992. Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase; identification of flavin and calmodulin binding sites. *J. Biol. Chem.* 267:10976–81
5. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. 1991. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351: 714–18
6. Bredt DS, Hwang PM, Snyder SH. 1990. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 347:768–70
7. Bredt DS, Snyder SH. 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* 86:9030–33
8. Bredt DS, Snyder SH. 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* 87:682–85
9. Bredt DS, Snyder SH. 1994. Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* In press
10. Buga GM, Griscavage JM, Rogers NE, Ignarro LJ. Negative feedback regulation of endothelial cell function by nitric oxide. *Circ. Res.* 73:808–12
11. Busconi L, Michel T. Endothelial nitric oxide synthase. N-terminal myristoylation determines subcellular localization. *J. Biol. Chem.* 268:8410–13
12. Craven PA, Derubertis FR. 1978. Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activitors by heme and heme proteins: evidence for the involvement of the paramagnetic nitrosyl-heme complex in enzyme activation. *J. Biol. Chem.* 253:8433–43
13. Förstermann U, Pollock JS, Schmidt HHHW, Heller M, Murad F. 1991. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* 88:1788–92
14. Furchgott RF. 1988. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor

is nitric oxide. In *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium*, ed. PM Vanhoutte, pp. 401–414. New York: Raven
15. Furchgott RF, Zawadski JV. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373–76
16. Garthwaite J, Charles SJ, Chess-Williams R. 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336:385–88
17. Garthwaite J, Garthwaite G, Palmer RMJ, Moncada S. 1989. NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur. J. Pharmacol.* 172:413–16
18. Giovanelli J, Campos KL, Kaufman S. 1991. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. *Proc. Natl. Acad. Sci. USA* 88:7091–95
19. Green LC, De Luzuriaga KR, Wagner DA, Rand W, Istfan N, et al. 1981. Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. USA* 78:7764–68
20. Green LC, Tannenbaum SR, Goldman P. 1981. Nitrate synthesis and reduction in the germ-free and conventional rat. *Science* 212:56–58
21. Gruetter CA, Barry BK, McNamara DB, Gruetter DY, Kadowitz PJ, Ignarro LJ. 1979. Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosamine. *J. Cyclic Nucleotide Res.* 5:211–24
22. Hevel JM, White KA, Marletta MA. 1991. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Chem.* 266:22789–91
23. Hibbs JB Jr. Vavrin Z, Taintor RR. 1987. L-Arginine is required for the expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* 138:550–65
24. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA* 84: 9265–69
25. Iyengar R, Stuehr DJ, Marletta MA. 1987. Macrophage synthesis of nitrite, nitrate, and N-nitrosoamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. USA* 84:6369–73
26. Deleted in proof
27. Kaufman S. 1993. New tetrahydrobiopterin-dependent systems. *Annu. Rev. Nutr.* 13:261–86
28. Klatt P, Schmidt K, Mayer B. 1992. Brain nitric oxide synthase is a heme protein. *Biochem. J.* 288:15–17
29. Knowles RG, Palacios M, Palmer RMJ, Moncada S. 1989. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. USA* 86: 5159–62
30. Kwon NS, Nathan CF, Gilker C, Griffith OW, Matthews DE, Stuehr DJ. 1990. L-Citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen. *J. Biol. Chem.* 265:13442–45
31. Lamas S, Marsden PA, Li GK, Tempst P, Michel T. 1992. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. USA* 89:6348–52
32. Leaf CD, Wishnok JS, Hurley JP, Rosenblad WD, Fox JG, Tannenbaum SR. 1990. Nitrate biosynthesis in rats, ferrets, and humans. Precursor studies with L-arginine. *Carcinogenesis* 11:855–58
33. Leone AM, Palmer RMJ, Knowles RG, Francis PL, Ashton DS, Moncada S. 1991. Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. *J. Biol. Chem.* 266:23790–95
34. Deleted in proof
35. Marletta MA. 1993. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* 268:12231–34
36. Masters BSS, Bilimoria MH, Kamin H, Gibson QH. 1965. The mechanism of 1- and 2- electron transfers catalyzed by reduced triphosphopyridine-cytochrome *c* reductase. *J. Biol. Chem.* 240:4081–88
37. Masters BSS, Kamin H, Gibson QH, Williams CH. 1965. Studies on the mechanism of microsomal triphosphopyridine nucleotide-cytochrome *c* reductase. *J. Biol. Chem.* 240:921–31
38. Mayer B, John M, Böhme E. 1990. Purification of a $Ca^{++}$/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. *FEBS Lett.* 277:215–19
39. Mayer B, John M, Heinzel B, Werner ER, Wachter H, et al. 1991. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxidoreductase. *FEBS Lett.* 288:187–91
40. Mayer B, Schmidt K, Humbert P,

Bohme E. 1989. Biosynthesis of endothelium-derived relaxing factor: A cytosolic enzyme in porcine aortic endothelial cells $Ca^{++}$-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochem. Biophys. Res. Commun.* 164:678–85

41a. McMillan K, Bredt DS, Hirsch DJ, Snyder SH, Clark JE, Masters BSS. 1992. Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl. Acad. Sci. USA* 89:11141–45

41b. McMillan K, Masters BSS. 1993. Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interaction. *Biochemistry* 32:9875–80

42. Michel T, Li GK, Busconi L. 1993. Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* 90: 6252–56

43. Mitchell HH, Schonle HA, Grindly HS. 1916. The origin of the nitrates in the urine. *J. Biol. Chem.* 24:461–90

44. Murphy MJ, Siegel LM. 1973. Siroheme and Sirohydrochlorin. The basis for a new type of porphyrin-related prosthetic group common to both assimilatory and dissimilatory sulfite reductases. *J. Biol. Chem.* 248:6911–19

45. Murphy JJ, Siegel LM, Kamin H, Rosenthal D. 1973. Reduced nicotinamide adenine dinucleotide phosphate-sulfite reductase of enterobacteria. II. Identification of a new class of heme prosthetic group: an iron-tetrahydroporphyrin (isobacteriochlorin type) with eight carboxylic acid groups. *J. Biol. Chem.* 248:2801–14

46. Narhi LO, Fulco AJ. 1986. Characterization of a catalytically self-sufficient 119,000-Dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* 261:7160–69

47. Narhi LO, Fulco AJ. 1987. Identification and characterization of two functional domains in cytochrome $P\text{-}450_{BM\text{-}3}$, a catalytically self-sufficient monooxygenase induced by barbiturates in *Bacillus megaterium*. *J. Biol. Chem.* 262: 6683–90

48. Ostrowski J, Barber MJ, Rueger DC, Miller BE, Siegel LM, Kredich NM. 1989. Characterization of the flavoprotein moieties of NADPH-sulfite reductase from *Salmonella typhimurium* and *Escherichia coli*. Physicochemical and catalytic properties, amino acid sequence deduced from DNA sequence of *cysJ*, and comparison with NADPH-cytochrome P-450 reductase. *J. Biol. Chem.* 264:15796–808

49. Otvos JD, Krum DP, Masters BSS. 1986. Localization of the free radical on the flavin mononucleotide of the air-stable semiquinone state of NADPH-cytochrome P-450 reductase using $^{31}P$ NMR spectroscopy. *Biochemistry* 25:7220–28

50. Palmer RMJ, Ashton DS, Moncada S. 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333:664–66

51. Palmer RMJ, Ferrige AG, Moncada S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524–26

52. Palmer RMJ, Moncada S. 1989. A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 158:348–52

53. Peterson JA, Prough RA. 1986. Cytochrome P-450 reductase and cytochrome $b_5$ in cytochrome P450 catalysis. In *Cytochrome P-450. Structure, Mechanism, and Biochemistry*, ed. PR Ortiz de Montellano, pp. 89–117. New York: Plenum. 556 pp.

54. Pollock JS, Förstermann U, Mitchell JA, Warner TD, Schmidt HHHW, et al. 1991. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA.* 88:10480–84

55. Pollock JS, Nakane M, Förstermann U, Murad F. 1992. Particulate and soluble bovine endothelial nitric oxide synthases are structurally similar proteins yet different from soluble brain nitric oxide synthase. *J. Cardiovasc. Pharmacol.* 20 (Suppl. 12):S50–S53

56. Porter TD, Kasper CB. 1985. Coding nucleotide sequence of rat NADPH-cytochrome P-450 oxidoreductase cDNA and identification of flavin-binding domains *Proc. Natl. Acad. Sci. USA* 82: 973–77

57. Pufahl RA, Marletta MA. 1993. Oxidation of $N^G$-hydroxy-L-arginine by nitric oxide synthase: evidence for the involvement of the heme in catalysis. *Biochem. Biophys. Res. Commun.* 193:963–70

58. Pufahl RA, Nanjappan PG, Woodard RW, Marletta MA. 1992. Mechanistic probes of N-hydroxylation of L-arginine by the inducible nitric oxide synthase from murine macrophages. *Biochemistry.* 31:6822–28

59. Radomski MW, Palmer RMJ, Moncada

S. 1990. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. USA* 87:10043–47

60. Ravichandran KG, Boddupalli SS, Hasemann CA, Peterson JA, Deisenhofer J. 1993. Crystal structure of hemoprotein domain of $P450_{BM-3}$, a prototype for microsomal p450's. *Science* 261: 731–36
61. Rogers NE, Ignarro LJ. 1992. Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. *Biochem. Biophys. Res. Commun.* 189:242–49
62. Schmidt HHHW, Pollack JS, Nakane M, Gorsky LD, Forstermann U, Murad F. 1991. Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase. *Proc. Natl. Acad. Sci. USA* 88:365–69
63. Schmidt HHHW, Smith RM, Nakane M, Murad F. 1992. $Ca^{+2}$/calmodulin-dependent NO synthase Type I: a biopteroflavoprotein with $Ca^{+2}$/calmodulin-independent diaphorase and reductase activities. *Biochemistry* 31:3243–49
64. Siegel LM, Kamin H. 1968. In *Flavins and Flavoproteins, Second International Conference,* ed. K Yagi, pp. 15–40. Tokyo: University Park Press
65. Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF. 1991. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. USA* 88: 7773–77
66. Stuehr DJ, Griffith OW. 1992. Mammalian nitric oxide synthases. *Adv. Enzymol. Rel. Areas Mol. Biol.* 65:287–346
67. Stuehr DJ, Ikeda-Saito M. 1992. Spectral characterization of brain and macrophage nitric oxide synthases. *J. Biol. Chem.* 267:20547–50
68. Stuehr DJ, Kwon NS, Nathan CF, Griffith OW, Feldman PL, Wiseman J. 1991. $N^{\omega}$-Hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.* 266: 6259–63
69. Stuehr DJ, Marletta MA. 1985. Mammalian nitrate biosynthesis: Mouse macrophages produce nitrate and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* 82:7738–42
70. Stuehr DJ, Marletta MA. 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *J. Immunol.* 139: 518–25
71. Wagner DA, Young VR, Tannenbaum SR. 1983. Mammalian nitrate biosynthesis: Incorporation of $[^{15}N]$ammonia into nitrate is enhanced by endotoxin treatment. *Proc. Natl. Acad. Sci. USA* 80:4518–21
72. White KA, Marletta MA. 1992. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* 31: 6627–31
73. Xie Q-W, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, et al. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256:225–28
74. Yasukochi Y, Masters BSS. 1976. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* 251:5337–44